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HEAT TOLERANCE AND THE PERIPHERAL EFFECTS OF
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SEATTLE DEPT OF MEDICINE (DERMATOLOGY)

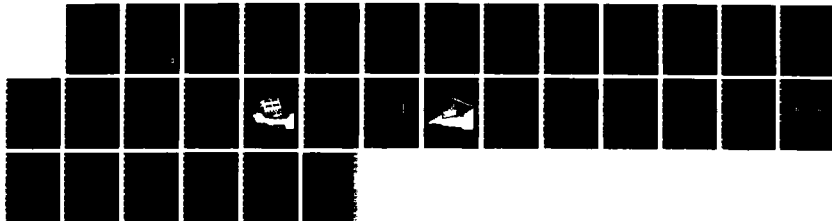
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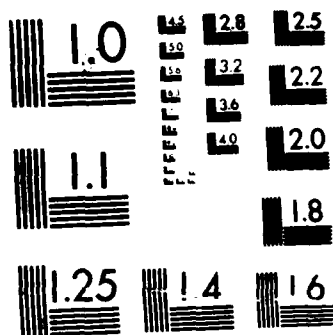
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HEAT TOLERANCE AND THE PERIPHERAL EFFECTS OF ANTICHOLINERGICS

- I. A non-invasive method for estimating the cholinergic sensitivity of the eccrine glands in humans.

ANNUAL SUMMARY REPORT

Kenneth K. Kraning, Sc.D.
Paul A. Lehman, B.S.
Roger G. Gano, B.S.
Terri S. Weller, B.S.

30 January 1985

Supported by

U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-83-C-3181

Departments of Medicine (Dermatology) and Environmental Health
University of Washington
Seattle, Washington 98195

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ABSTRACT

Chubley et al. [1] proposed that measurement of forearm sweat responses to graded intradermal injections of acetylcholine can be used as a sensitive index of anticholinergic drug potency. Independently, we developed a human sweat gland assay differing from theirs in several important respects: It stimulates larger, well-defined areas; it uses non-invasive iontophoresis [2] instead of intradermal injection for administration of mecholyl; it is comprehensive, and measures both active gland density and the time course of sweat production simultaneously on four adjacent test sites [3] instead of only total glands responding; and antagonists can be administered to skin sites by iontophoretic pretreatments and their effects on mecholyl dose-response relationships directly measured.

By Faraday's Law, the total dose of ions delivered to skin is proportional to the product of current density and duration of iontophoresis. This defines the highest possible dose, i.e., the condition in which only drug ions are available for transport. In these studies current density and duration were kept constant at 0.132 mA-cm^{-2} and 180 sec, respectively, and the maximum drug transfer was 250 nMol-cm^{-2} . Lesser doses are produced with the same current-time combination by diluting drug solutions with equimolar NaCl solution: The total number of ions transported is always the same but the proportion of drug ions varies. From over 300 individual test replications in more than 35 subjects, we conclude that this assay method can be used safely in normal subjects without peripheral or systemic side effects. Typical mecholyl log dose-response curves are produced for both sweat rate and active gland density over a dose range of 0.4 to 250 nMol-cm^{-2} mecholyl [4].

FOREWORD

For the protection of human subjects, the investigators have adhered to policies of applicable Federal Law 45CFR46.

"Soluene-350" is a registered trademark of Packard Chemical Company.

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These studies were approved by the University of Washington Human Subjects Review Committee.

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INTRODUCTION

In clinical studies of anticholinergic efficacy in humans in which either basal level of parasympathetic tone or heat stress is used as a stimulus, it is difficult to estimate potency directly because the acetylcholine level being antagonized is unknown. Clinical studies are further confounded by questions of metabolism, bioavailability, and pharmacokinetics [5-12]. Direct potencies of cholinergic agonists and antagonists are often determined from *in vitro* dose-response assays on isolated tissues suspended in organ baths, where the concentration of drugs can be carefully controlled [5,13,14]. Yet because there are no animal models that can substitute perfectly for human responses, studies on human subjects must always form the bases for decisions about dosage and drug of choice [1,5,15]. It would be useful, therefore, to supplement findings of conventional clinical studies with data from a direct human assay method that would have the precision of the organ bath but could be used *in situ* on a sensitive muscarinic end organ. Requirements for a direct human cholinergic dose-response assay are (1) that it can be used safely *in vivo* without pain or aftereffects, (2) that it be quantitative, and (3) that it measure responses of agonists and antagonists applied directly to an accessible muscarinic end organ, without metabolic or pharmacokinetic complications. Further, the muscarinic end organ (4) should not have substantial basal activity, as this could interfere with the assay. Preferably, the assay would be (5) non-invasive and (6) suitable for application in screening studies of normal subjects or clinical studies of outpatients. A direct assay might also be useful for obtaining directly relevant human data on specific, applied problems, such as those encountered in military medicine, that are too difficult or hazardous to obtain by other experimental approaches.

The eccrine glands seem an ideal *in vivo* end organ system for direct cholinergic assay. They constitute a dense ($150-300 \text{ cm}^{-2}$), distributed-organ network over the general skin surface. Although they are innervated by postganglionic fibers of the sympathetic nervous system, their active neuroglandular unions appear to be predominantly cholinergic and muscarinic [5,16]. Sweat glands are accessible for stimulation and measurement from the skin surface. Studies *in vitro* have well characterized the responses of isolated human glands to cholinergic agonists and antagonists [13]. It has been shown that sweat gland activity is at least as sensitive as that of salivary glands to the effects of systemic atropine sulfate and is apparently much more sensitive than other non-invasive measures, such as heart rate, pupil size, and near-point visual accommodation [1].

Chubley et al. first proposed an *in vivo* sweat gland assay [1]. They found that measurement of forearm sweat responses to graded intradermal injections of acetylcholine (ACh) was a sensitive index of the anticholinergic action of systemically administered atropine sulfate. However, their assay used intradermal injection, an invasive procedure that stimulates only a small area whose size and shape are difficult to control and whose depth is difficult to reproduce. Their assay provides no method for directly administering antagonists to the sweat glands. Its only measurement is a total count of the number of glands responding, including those lying at and beyond the borders of the stimulated area: Secretion rate is not determined.

Independently, we also set out to develop an *in vivo* assay method based on the responsiveness of the eccrine sweat glands [2]. Our approach is more biophysical

than that of Chubley's group, which we feel gives our method some significant advantages. First, ours is non-invasive. Skin areas are stimulated with cholinergic agonists rapidly, painlessly, and safely, without needle puncture or trauma, by iontophoresis (the transportation of minute quantities of cationic agonists and/or antagonists across unbroken skin, using the force of a weak DC electric field). Stimulated skin areas are well defined with this technique, and the stimulated area is larger than the measurement area so that stimulation border effects do not influence the data. Second, we can administer antagonists as well as agonists by iontophoresis in order to directly measure their inhibitory action on sweat gland responses. Third, we use more sophisticated measures: The sweating rate, derived from an assembly of 4 evaporative capsules with specially designed electronics, is our primary measurement [3]. Sweat counts are determined on per cm^2 of skin with iodinated paper, which has advantages over plastic impression material for quantitation and storage.

Development of this assay was the main focus of the first year's contract work. A major problem was that iontophoresis had previously been used only as a qualitative tool for stimulating or blocking the sweat glands [16-18]. A theoretical basis for quantitation of the method exists, however, and we felt that through further study a sufficiently accurate way to implement quantitative iontophoretic drug delivery could be found.

BACKGROUND

A safe, non-invasive, human assay of eccrine sweat gland responsiveness to muscarinic agonists and antagonists can be applied to problem areas of direct relevance to military medicine. This section addresses these applications.

Anticholinergic Assays

Poisoning by insecticides or chemical agents having anticholinesterase activity is usually treated with compounds that inhibit the activity of ACh on muscarinic cholinergic effector sites: Atropine sulfate is the prototype [5]. In evaluating the antimuscarinic properties of various compounds, a number of factors must be considered, including their pharmacokinetics and bioavailability. Of equal importance is a comparison of the ability of antagonists to inhibit the action of muscarinic agonists once they reach the target tissues. It has been customary to make such comparisons on animal tissue *in vitro*. The degree to which graded stimulation of a sample tissue by muscarinic agonist is inhibited by different doses of antagonists is often presented as a family of log dose-response plots from which estimates can be made of threshold, sensitivity, and potency [5,13]. Such analyses are important for developing new antidotal compounds and for studying their mechanisms of action. Ultimately, however, the direct relevance of animal studies to humans always must be demonstrated, and large differences in treatment efficacy have been observed between animal species because of non-specific binding of drug to tissues [15]. Thus, a safe and sensitive human muscarinic assay would be a very useful addition to the existing battery of bioassay techniques. The rate of sweat production by the eccrine glands on non-glabrous skin regions of humans is highly sensitive to the actions of muscarinic agonists and antagonists; depression of stimulated sweating can be used, therefore, as a sensitive indicator of the peripheral antimuscarinic activity in antidotes to anticholinesterases [16]. Several previous studies

have used heat stress to stimulate sweating in volunteers and measured the depression in sweating induced by systemically administered atropine sulfate as an index of antimuscarinic activity [8-12]. But these kinds of experiments are extremely limited in the amount of data that can be obtained from each subject and are expensive and time-consuming. Development of standard iontophoretic techniques for stimulating glands on small skin areas with muscarinic agonists and, at the same time, blocking this action with antagonists (administered either systemically in staircase fashion or by iontophoresis pretreatment of the test site) would allow multiple dose-response comparisons to be made safely among different anticholinergic substances on the same subject.

Thermoregulation

The primary physiological function of the eccrine sweat glands in humans is to provide a means for heat loss by evaporation; in fact, they provide the only significant physiological defense against heat stress in jungle and desert climates. The possibility of untimely administration of chemical agent antidotes cannot be ruled out. If they are injudiciously administered under conditions of jungle or desert warfare, even a well-acclimated soldier's performance could be severely compromised by hyperthermia; for the less fortunate, fatal heat stroke could result. Quantitation of decrements in heat tolerance caused by chemical agent antidotes in different doses is a longstanding problem of military medicine [6-12] but data are sparse because of the inherent difficulties in administering high doses of antidotes to heat-stressed subjects. A non-invasive assay of sweat gland responsiveness to graded muscarinic stimulation by iontophoresis, performed in resting subjects given staircase doses of antagonist, would be a way of obtaining a family of dose-response curves from each volunteer. The assay could be done in the safety of a room temperature environment.

Heat Tolerance

Heat-training protocols have often been proposed for rapidly acclimating workers and military personnel in temperate zones who must be transported abroad for heavy work or combat in tropical or desert climates. Administration of supervised heat-training protocols are expensive and time-consuming, and some persons will benefit little because they are either heat-intolerant, i.e., more prone to heat stroke, or because they are already well acclimated. A rapid method for screening and grading the heat tolerance of large numbers of persons would be beneficial in these situations, especially for identifying those who are heat-intolerant. Shavartz et al. [19], have proposed an empirical room-temperature exercise protocol for indexing heat tolerance, but this procedure requires constant supervision and measurement of heart rate and rectal temperatures. Others have proposed that sweating rate may be related to heat tolerance [20], and Collins et al. demonstrated a promising method, not further pursued, that related state of acclimation to the maximal responsiveness of sweat glands to local cholinergic stimulation [21]. Again, the assay of sweat gland responsiveness to muscarinic stimulation by iontophoresis would be a way of obtaining complete dose-response data from each volunteer. If these data correlate well to heat tolerance as determined by more conventional thermal tests, then it may be possible to develop a simple screening test based solely on a volunteer's sweat responses to graded muscarinic stimulation.

RATIONALE

We had developed instrumentation for quantitating the sweating responses of several small skin areas simultaneously treated by single-dose iontophoresis, and thought that it might be possible to apply these techniques to the above problems. Although theory suggested that iontophoresis could be developed into a variable dose method, i.e., that there is a relationship between the parameters of iontophoresis (current and time) and the dose delivered into the skin, experimental documentation for this relationship has been lacking. The main purpose of the first year of this study was to develop iontophoresis as a quantitative investigative tool for obtaining dose-response data in human subjects. Quantitative studies using the techniques developed were planned in each of the three areas outline above. These studies were to be completed by the end of the second contract year.

GOALS

The goal of the first year of this study was to develop iontophoresis and sweat measuring techniques into a non-invasive quantitative assay method that could be used safely on very small skin areas in humans. This utility of this assay will be demonstrated by experimental application to several problems of scientific and military medical importance.

OBJECTIVES

The overall objectives of this study are: (1) To develop technology for obtaining accurate and reproducible dose-response data non-invasively from the eccrine sweat gland system, (2) To compare effectiveness of different locally applied cholinergic muscarinic antagonists in altering dose-response patterns of eccrine glands to locally applied cholinergic agonists, (3) To compare cholinergic sensitivities of individuals' eccrine glands to their performance scores on standard heat-tolerance tests, (4) To study the time course of changes in cholinergic sensitivity of the eccrine glands during acclimation to heat, and (5) To compare the effect of locally and systemically applied antagonists on responses to locally applied agonist.

METHODS

A major part of the work during this first contract year was developmental rather than investigative, and was centered around the iontophoretic dose-response assay method. These tasks involved trial and error testing and modification of the apparatus, procedures, and techniques. The apparatus and procedures finally adopted are described. They were used to obtain the data presented in the Results section.

Iontophoresis

Regions. Our instrumentation (Figure 1) was designed to study simultaneously four adjacent treatment sites on regions of the proximal volar surface of the forearm. The proximal half shows less variance and it is

difficult to secure intimate contact of apparatus over the tendinous areas adjacent to the wrist. The test sites are of circles 2.1 cm diameter in a line with centers 2.6 cm apart. The 4-site region under study is defined by a rectangular plastic template secured by Velcro straps around the forearm. The proximal volar surface of the average male supinated forearm is large enough to define 2 side-by-side regions on each forearm without overlap. This provides as many as 16 assay sites per volunteer during a study session. Slight flexion or rotation of the forearm will cause shifts in the skin surface underlying the template; alignment should be checked frequently. In most female subjects only 1 region per forearm can be defined.

Apparatus. The anode assembly consists of a machined 11.5 cm x 3.7 cm Plexiglas block containing four 0.9 cm D Ag/AgCl buttons removed from disposable electrocardiogram (ECG) monitoring electrodes ("Red Dot" No. 2249, Medical Products Division/3M, St. Paul, MN 55144). Glass fiber filter pads of 2.1 cm D (Whatman Grade GF/B), holding 0.22 ml of pretreatment or treatment solution, sit snugly over the anode buttons in machined wells of 2.1 cm D and 0.1 cm deep. Greater volumes will cause leakage of the conducting electrolyte between adjacent anodes. The original anodes were copper and resulted in uneven sweat stimulation, as evidenced by sweat prints. This problem was virtually eliminated by changing to Ag/AgCl anodes. The cathode assembly consists of 4 ECG electrodes mounted on the forearm extensor surface, parallel to and directly opposite the test sites. Each anode and each cathode are connected to independent battery-operated power sources (Figure 2) that maintain a constant current flux despite changes in resistance occurring because of polarization or hydration of the skin surface. Completely independent power sources and cathodes reduce the probability of significant interanodal currents.

Solutions. Stock solutions of NaCl and acetyl- β -methacholine are prepared at a concentration of 20 mM in twice-distilled, deionized, bacteria-free water (Sigma Chemical Company, St. Louis, MO 63178). Measured amounts of drug stock solution are diluted with 20 mM NaCl stock solution (Figure 3) to prepare a series of pretreatment or treatment solutions in which the total ion concentration is the same (20 mM) but the relative proportion of active drug ion is adjusted from 0.1% to 100%. From threshold to SRmax requires a 10^3 to 10^4 increase in agonist dose [13,22]. Changes in iontophoresis current cannot span this dose range. With the "ratio method," drug dilutions with NaCl can be made quite accurately over a 10^4 -fold range.

Calculations. From Faraday's Law of Electrolysis, the molar quantity of cationic material transported across the pad-skin boundary is proportional to the total charge moved (current x time) and to the ratio of drug to total ions in the solution-soaked pad. This quantity is given by:

$$Q = (\alpha \cdot I \cdot t) / (z \cdot F)$$

where Q is the predicted quantity of drug cations transported in nMol-cm⁻², α is the molar fraction of drug ions in the iontophoresis solution, I is the current density in mA-cm⁻², t is the duration of iontophoresis in sec, z is the charge number of the cation, and F is Faraday's constant ($9.65 \cdot 10^{-2}$ mA-sec-nMol⁻¹). In both the dose-response tests and antagonist pretreatments, I and t are first chosen so that the highest desired dose will just be obtained using pure drug solution, i.e., $\alpha = 1.0$. The same values for I and t are used

on all sites. Lower doses are obtained by using the solutions with smaller α values (Figure 3).

Procedures

Pretreatment. If agonist iontophoresis is attempted on unconditioned skin, the sweat glands often are not stimulated as expected. This problem has been noted by others and may occur because the skin, initially dry, exhibits an extremely high electrical resistance [23,24]. We found that agonist iontophoresis is optimized by a "conditioning" pretreatment: iontophoresis of the same sites with 20 mM NaCl solution alone. By hydrating the stratum corneum, pretreatment reduces the electrical resistance and permits appropriate current levels to be maintained with low voltages during subsequent agonist iontophoresis. Pilot tests showed that NaCl pretreatment at 0.06 mA-cm^{-2} for 240 sec produces an adequate conditioning effect. Simply moistening the skin with electrolyte is not as effective as iontophoresis.

Treatment. Following pretreatment, the skin area inside the template is blotted dry and the iontophoresis procedure is again applied to the same sites, using dilutions of acetyl- β -methacholine. In both the pretreatment and treatment stages, the same procedure is followed: The anode assembly is placed into the template and the currents are rapidly adjusted to the prescribed level and held at this level for the prescribed time. We have used current densities from 0.06 to 0.15 mA-cm^{-2} and durations from 30 to 360 sec.

Sweat Measurements

Sweat Rate. Immediately after agonist iontophoresis, 4 small circular evaporative sweat capsules whose centers align with the treatment centers are placed on the sites and secured to Velcro fasteners on the template guide. The skin area exposed inside each capsule is 2.85 cm^2 , purposely smaller than the treated area, to eliminate border effects of untreated skin. The sweat capsules use fast-responding capacitance relative humidity sensors (Figure 4); details of their design and construction are fully described elsewhere [3]. Briefly, dry nitrogen gas at 200 ml-min^{-1} is directed uniformly onto the skin surface inside each capsule. At 200 ml-min^{-1} , the capsule environment is completely exchanged every 2.3 sec. Initial tests showed this to be adequate for complete and rapid evaporation. The effluent nitrogen and evaporated water vapor mixture then passes into an upper chamber containing relative humidity and temperature sensors. Analog voltages corresponding to the measured relative humidity and temperature are automatically digitized every 5 sec and stored by a microcomputer on disk for future detailed analysis. Data are also processed on-line and displayed on a video monitor. Measurements of baseline transepidermal water loss are obtained before the start of the experiment. These minor corrections (0.01 to $0.03 \text{ mg-cm}^{-2}\text{-min}^{-1}$) are automatically subtracted by the computer from succeeding readings.

Active Gland Density. In most experiments the sweat capsules are removed soon after peak sweat rates are achieved, the skin is quickly blotted with tissue, and an impression of active glands is made using iodized paper. Dessicated xerography paper is cut into 1 by 6 inch strips and hung for 24 to 36 hr in a chamber containing a few grams of iodine crystals in an open dish. Prepared strips are fastened onto the holder (Figure 1), and steady and even

pressure is applied for 10 to 20 sec, depending on the sweat rate. Paper starch reacts with iodine wherever the two are solubilized by a sweat droplet. Prints will keep for years if stored away from light in plastic 35 mm negative sleeves. Subsequently, assessment of active glands under each capsule area is made with the aid of a low-power surface microscope, a calibrated grid that subdivides the capsule area, and a computer program that tallies the grid counts.

Subjects

All subjects were healthy adult Caucasian volunteers, mainly students, recruited by advertisement in the surrounding university community. They were informed of possible risks and they freely consented to participate in these studies. Excluded were those having any chronic disease, allergy, family history of allergy, or evidence of acute illness on the day of the experiment. Records were kept of their heat stress and exercise history for future data segregation. All tests were performed in an air-conditioned laboratory with subjects in a thermally neutral state.

RESULTS

Example Assay Data

Data selected from one male subject are shown in Figure 5 to illustrate raw data obtained in the mecholyl iontophoresis assay. Typically, peak sweating rate occurs about 8 to 10 min following the end of iontophoresis. In this early experiment, iontophoresis time was 30 sec and the current was 0.058 mA-cm⁻² on all sites. Mecholyl dose was varied by adjusting α , and ranged from 0.2 to 17 nMol-cm⁻² of skin surface. For most subjects a dose greater than 17 nMol-cm⁻² is needed to obtain SR_{max}; in this experiment we may have failed to obtain the subject's true SR_{max}. Our conditions are now standardized at 0.132 mA-cm⁻² and 180 sec, which gives a maximal dose of 250 nMol-cm⁻². Sweat rates, expressed as a percent of the highest sweat rate observed, vary hyperbolically with calculated dose (Figure 6a). Figure 6b, a log dose plot of the same data, demonstrates that this method of iontophoretic dose adjustment yields sigmoidal response curves expected of dose-response assays [5]. Log dose-response curves were fitted to data by Foster's least-squares method [22].

As soon as peak recordings are completed, sweat prints of the region are made (Figure 5); at the same time, capsules are transferred to the region where iontophoresis has just been completed. We define a gland as above threshold if it appears on a sweat print, and we assume the maximal active gland density to be that observed at peak sweat rate. Sweat print counting errors are highest at maximal sweat rate because of overlaps in the secretion area of two or more glands. Shorter imprint times are sometimes useful. Figure 6c is a normalized plot of log dose vs. percent of active glands above threshold. Over this dose range, the sweat rate and gland count curves have nearly identical shapes, although the latter has somewhat more scatter. The slope of the cumulative percent curve is bell-shaped and can be thought of as the distribution of thresholds about dose (Figure 6d). Mean threshold dose corresponds to the ED₅₀.

Regional Variation

The proximal region of the flexor forearm surface constitutes the experimental field. The capsules are small enough to allow 2 side-by-side experiments on each forearm. Thus, sixteen dose-response tests can be performed in a single subject session. We attempted to determine whether variation in responses between forearm skin regions is random or consistently related to arm or to aspect. In 7 male subjects we determined responses to 1 very low and 3 very high mecholyl doses on the 4 forearm regions. Corresponding sites on the forearm regions received identical mecholyl treatments: site 1 - 1 nMol-cm⁻², site 2 - 100 nMol-cm⁻², site 3 = 160 nMol-cm⁻², and site 4 = 250 nMol-cm⁻². This protocol is now standard for all subjects and is performed on region A. It allows us to prejudge the dose yielding a maximal response and to adjust the dose-response assay accordingly. Figure 7 is a plot of means and SEMs of peak sweat rate and active gland counts for the 4 regions, grouped by dose. In terms of active glands, region d was consistently, but not significantly, higher than the other 3 regions. This was not true for peak sweating rate. The 250 nMol-cm⁻² dose (site 4 on all regions) gave more variable, but not significantly different, sweating rates compared to those of the other 3 doses. No significant differences in either peak sweat rate or active gland density were found among the 3 highest doses on any skin region. Variability was lower for both parameters at 1 nMol-cm⁻² than at the 3 higher doses.

Current and Duration

Although Faraday's Law predicts that combinations of current and time yielding equivalent charge transfer should transport the same number of ions into the skin, the velocity of ionic migration is related not to charge transfer but to the potential drop between the source (anode) and the sweat glands. Therefore, assuming the skin is an ohmic conductor, a higher velocity would be expected at higher current levels. Thus, a combination of low current and long time might produce a significantly different distribution of drug cations around the sweat glands than an equivalent charge transfer from a high current short time combination. To study this, we compared the responses in 5 male subjects to mecholyl iontophoresis on 4 left arm sites, using 4 different time-current combinations giving the same charge transport (13.9 mC-cm⁻²) and total dose (16 nMol-cm⁻²). They were 0.029 mA-cm⁻² for 480 sec, 0.058 mA-cm⁻² for 240 sec, 0.116 mA-cm⁻² for 120 sec, and 0.173 mA-cm⁻² for 80 sec (Figure 8a). There appeared to be slight fall-off in peak sweat rate as current density was increased and duration was reduced, but this change was not significant (Figure 8b). Corresponding sites on the right arm were used as controls and were all treated at 0.029 mA-cm⁻² for 120 sec. There was no trend of change in these control areas nor was there a significant difference between them (mean = 6.45 mg-min⁻¹cm⁻²). Time of peak response (end of iontophoresis = time zero) was inversely related to the log of iontophoresis duration (Figure 8c).

Long-Term Changes

We restudied 6 male subjects a number of times over a 3 to 4 month period, in experiments in which different conditions of time and current were used. The pooled dose-response data are compiled for each subject in Figure 9. For the most part there is a reasonable agreement in the dose-response data over several

months in spite of different currents and iontophoresis times, although some subjects showed much more variability than others. Subjects who showed the most consistent responses within an experimental session tended to show the most consistency in response among experimental sessions.

In Vitro Studies

To determine the feasibility of using *in vitro* studies to obtain more exact knowledge of drug transport during iontophoresis, pilot tests were done on 5 samples of human abdominal skin obtained at autopsy. Iontophoresis was performed with ^{14}C -lidocaine-HCl. Lidocaine is of comparable size and weight to meholyl and should have the same order of electrophoretic mobility. Current density was 0.29 mA-cm^{-2} for 30 sec on 4 samples; no current was applied to the 5th sample, which served as a diffusion control. Following iontophoresis, the sample epidermis and dermis were separated by heating between two 60°C aluminum plates for 30 sec. Samples 1-4 were split at 2, 6, 10, and 20 min after iontophoresis, respectively; the control at 22 min. Epidermal and dermal samples as well as anodal filter pads containing the remaining labeled lidocaine and cathodal saline-soaked pads were placed in individual vials, digested with Soluene-350 (Packard Chemical Co.) and counted in scintillation fluid. Results, shown in Figure 10, indicate that (1) an extremely small amount of ^{14}C -lidocaine, within the limits of counting accuracy, was transferred from the top pad into the skin by simple diffusion alone (sample 5); (2) there was no trend of difference among samples 1-4, indicating insignificant diffusional transfer during the 20 min period following iontophoresis; (3) an average (samples 1-4) of only 1% of label reached the subdermal pad; (4) an average of 9% of the delivered dose reached the dermis; (5) the calculated total amount transferred was less than the observed amount; and (6) 90% of the delivered dose remained in the epidermis. Juhlin [23] observed that the "epidermal pool" of drug remains for several days after iontophoresis. Subsequent iontophoresis of NaCl solution over the test site reinduces substantial activity. We also have observed this storage and allow 2 to 3 weeks before retesting at the same site; this delay accounts for the normal shedding and rejuvenation cycle of the stratum corneum.

DISCUSSION

The improvements in methodology and technique during the first year of this study have made the non-invasive assay a sensitive, quantitative, and reproducible method. Our experience with well over 500 applications of iontophoresis in the past several years has been that the method is safe, painless, and devoid of any systemic aftereffects.

From the Faraday calculation, our range of total delivered dose is from about 0.2 to 250 nMol-cm^{-2} of skin surface. Corresponding agonist concentrations at the secretory cells is unknown and depends on the biophysics of iontophoretic transport. The skin is not a homogenous medium; most of the resistance to current (ion) flow is in the outermost skin layer, the stratum corneum, which is only $20 \mu\text{m}$ thick. Even when well hydrated, the stratum corneum has a resistivity 100 to 1000 times greater than that of the underlying viable epidermis and dermis [23]. It follows from this that the voltage (and thus the migration velocity) must fall sharply in transition from stratum corneum

to deeper tissues. Since the rate of mass transfer is fixed by the constant iontophoretic current, it is predicted that most ions would accumulate in the stratum corneum and only a small fraction would reach the sweat gland secretory cells. Our pilot experiment with labeled cation (^{14}C -labeled lidocaine-HCl) iontophoresis in cadaver skin confirmed this: 90% of ions remained within the epidermis and only 9 to 10% reached the dermis during iontophoresis. However, no significant diffusion occurred from epidermis to dermis following iontophoresis. More studies of this kind will be needed to determine the exact nature of the relationship between calculated dose and periglandular concentration; whether this relationship varies significantly among drugs, currents, and durations; and whether the sweat glands themselves are preferential pathways for ionic transport. Without this biophysical information, the assay results themselves must be relied upon as an indicator of method precision. The consistency of responses and small standard errors of group averages strongly indicate that periglandular concentration is a relatively constant fraction of total delivered dose. This was somewhat surprising to us because errors include variation due to subject, forearm region (Figure 7), and measurement as well as variation due to drug delivery.

The parallel between peak sweat rate and active gland density has been striking in all of our studies, although active gland density tends to reach a maximum at a somewhat lower dose than does peak sweat rate. It must be emphasized that our measurements of active gland density count only the number of glands above their respective thresholds (and the threshold of the method) and do not quantify their individual secretion rates. Given this, the calculation of average output per gland (peak sweat rate/active gland density) as a function of dose would be questionable because it would assume a normal distribution of glandular outputs. Less questionable is the estimation of mean threshold dose, which is the same as the ED_{50} on the log-dose vs. active gland density curve (Figure 6d). Because of the parallel between peak sweat rate and active gland density, it may be possible to simplify the assay method under certain conditions by eliminating measurement of sweating rate.

CONCLUSION

Ion-dilution iontophoresis was used with a single set of time and current conditions to achieve quantitative delivery of cholinergic agonist to sweat glands over the 1000-fold range needed to span threshold to maximal responses. Both peak sweating rate and active gland density varied hyperbolically related with the log of agonist dose. There was reasonable agreement in dose-response characteristics of individual subjects in experiments repeated over a period of 3 months. Subjects who showed the most consistent responses within an experiment also showed the most consistent responses between experiments.

RECOMMENDATIONS

A. Our non-invasive cholinergic assay method has proved to be both safe and quantitative. We can recommend its use to others who may wish to include such measurements in their studies provided that they (1) adhere to our specifications for electrode design, (2) use independent battery-operated constant current sources, (3) keep within our specified constraints for current density and

duration (the most comparable results will be obtained if current density is fixed as 0.132 mA-cm^{-2} , duration at 180 sec, and dose varied by ion dilution), (4) precede treatment iontophoresis with a conditioning step, and (5) use only proximal forearm areas and skin that is free of disease, cuts, or abrasions.

B. We have consistently used mecholyl as agonist in these studies because it is less susceptible to enzymatic degradation than ACH and provides longer periods of peak sweat rate [5]. Other muscarinic agonists that are even less susceptible to enzymatic action, such as pilocarpine, bethanachol, and carbachol, may be more suitable for the assay. We recommend that these compounds be compared with mecholyl.

C. Although the results on many subjects lead us to believe that iontophoretic delivery is fairly uniform among drugs and subjects, we recommend further studies on the biophysics of iontophoresis to confirm this point. The findings of these studies could lead directly to improvements in the assay. Indirectly, they could aid other investigators who might consider using iontophoresis in studies of skin blood flow or as a means for therapeutic drug delivery. Specifically, the progress of ^{14}C -labeled compounds through samples of fresh human skin, obtained at autopsy or from amputee donors, should be monitored by frozen-section radioautography and by scintillation counting of digested horizontal sections of dermis. From these studies it should be possible to construct an empirical model of the iontophoresis process from which periglandular concentration could be predicted from delivered dose.

D. McCance and Purohit [24] demonstrated ethnic differences in the number of sweat glands responding to a single dose of pilocarpine. This finding in itself does not reveal whether there are different maximal gland densities. Ethnic differences should be restudied with the non-invasive assay to clarify this point.

FIGURES

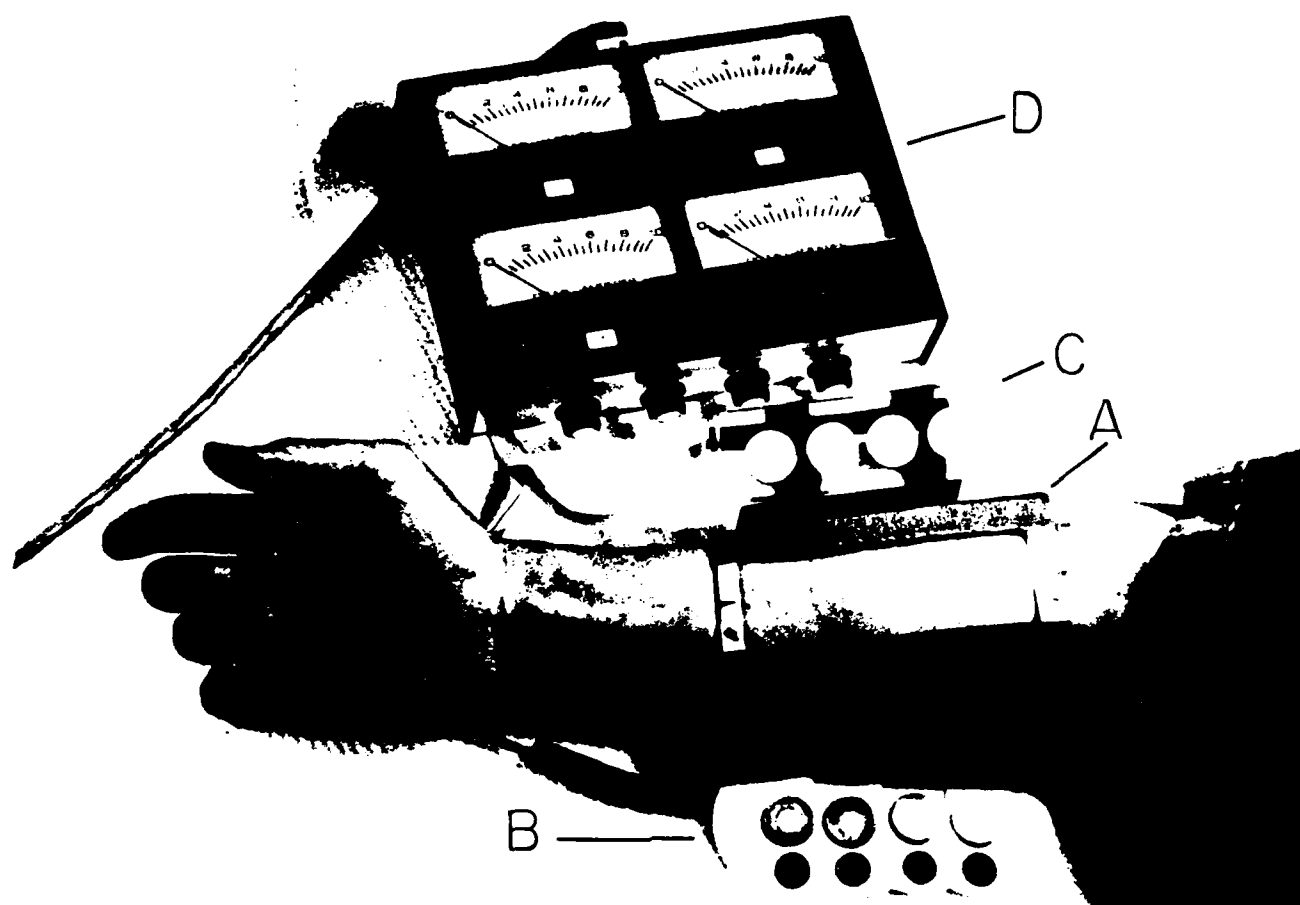


Figure 1. Iontophoresis apparatus. Test sites were 2.1 cm \varnothing circles 2.6 cm apart. (A) Plastic template defining the test region on flexor forearm surface. (B) Cathode assembly before attachment to extensor surface of forearm. (C) Ag/AgCl anode assembly topped with filter disks containing iontophoresis solution. (D) Control box containing 4 constant-current sources.

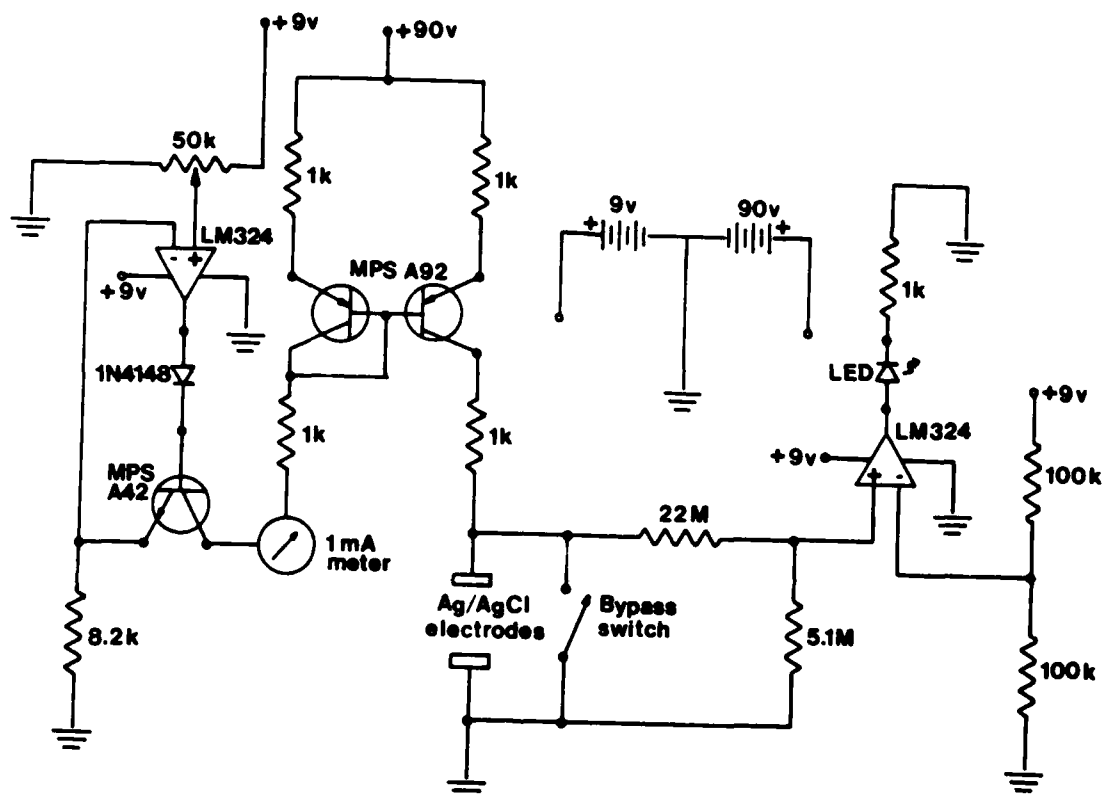


Figure 2. Schematic of constant current source driving each iontophoresis electrode pair. Power sources are 9 and 90 volt batteries. Current is proportional to voltage at 50K potentiometer. Light-emitting diode (LED) is activated if voltage drop is >20 V. This occasionally occurs during the first few seconds of pretreatment. Activating bypass switch immediately brings skin potential drop to 0 V.

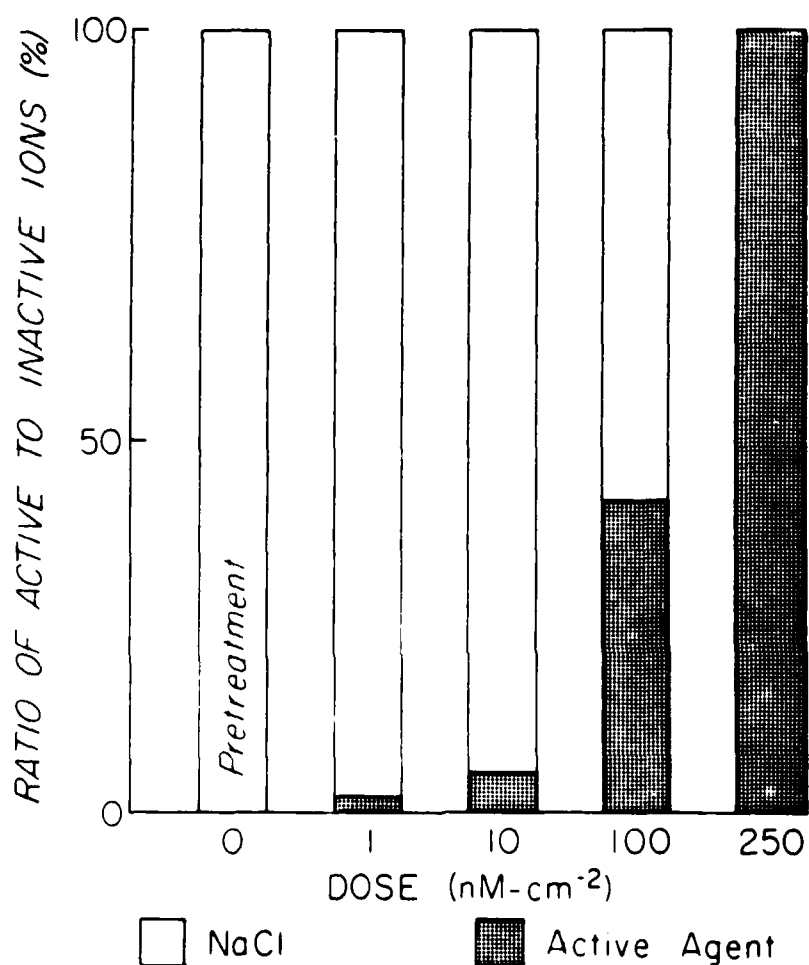


Figure 3. Adjustment of mecholyl dose by ion dilution. Using Faraday's Law, the current density and duration are back-calculated from the desired maximal dose, in this case, 250 nMol-cm⁻². This dose is obtained using a 20 mM iontophoresis solution containing 100% drug ions ($\alpha=1$). Smaller doses are obtained by diluting this solution with 20 mM NaCl. Dose is related to percentage of charge carried by drug cations, not to absolute molarity of drug solution.

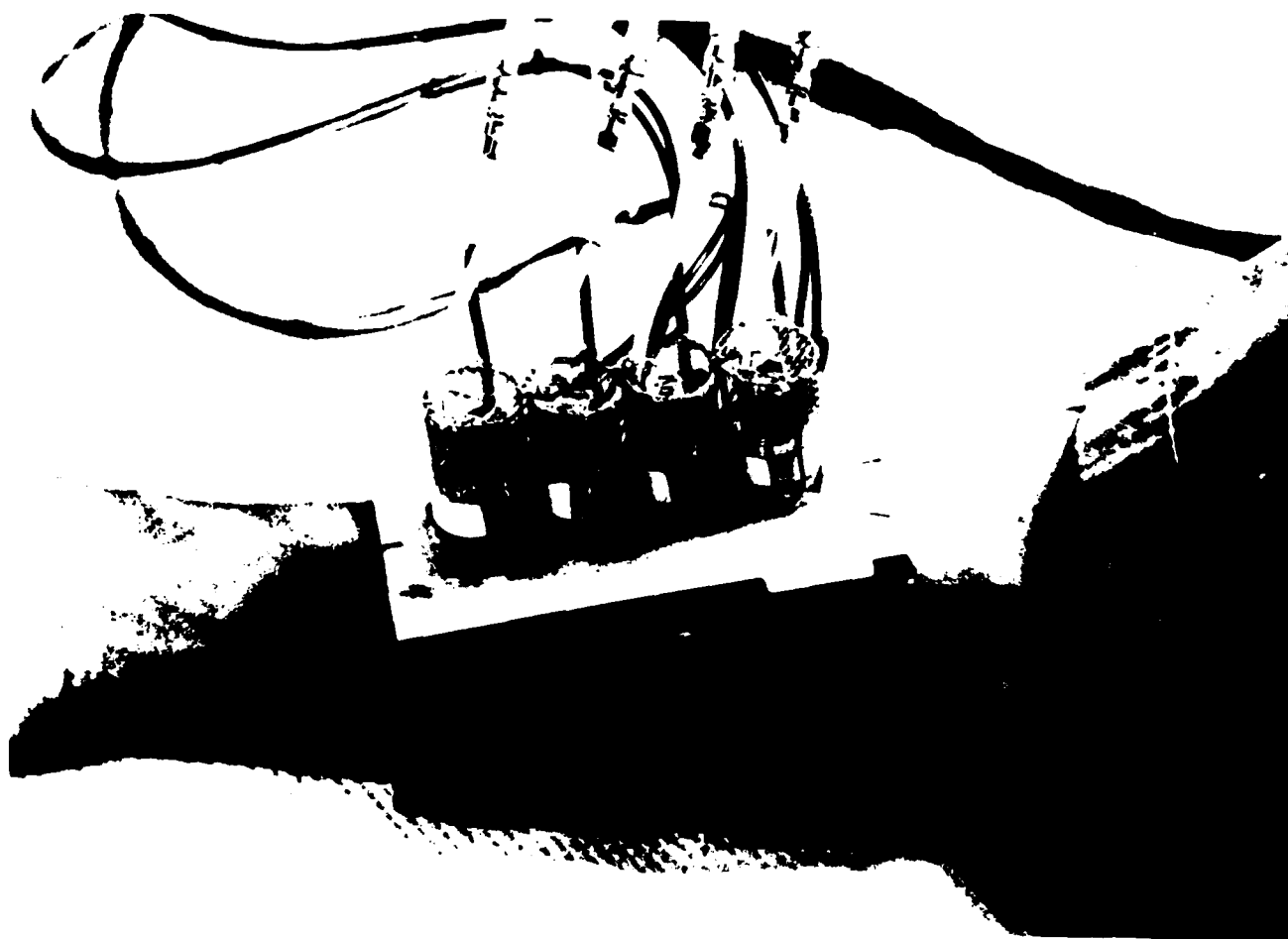


Figure 4. Evaporative capsules positioned on template during measurement of sweating rate.

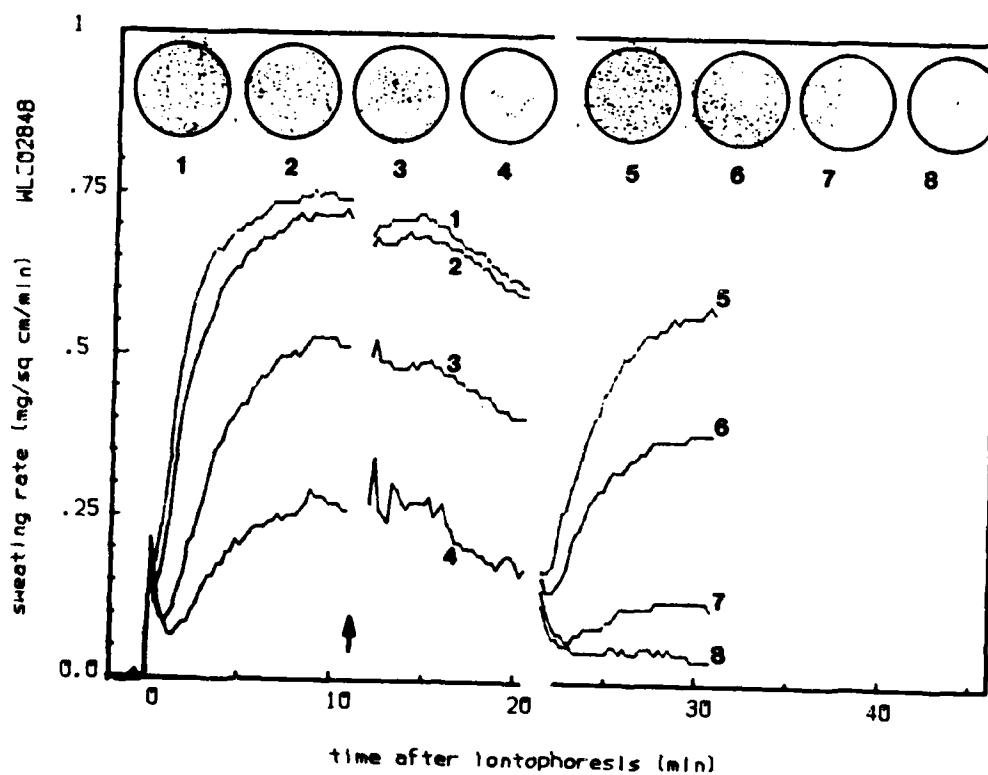


Figure 5. Computer plot of assay data on one subject. Two regions were used, one on each arm. Xerographic reproductions of sweat prints corresponding to each site appear at top of plot. Doses in nMol-cm^{-2} . Arrow indicates time of sweat prints 1-4. Capsules were then returned to sites while iontophoresis proceeded on contralateral arm.

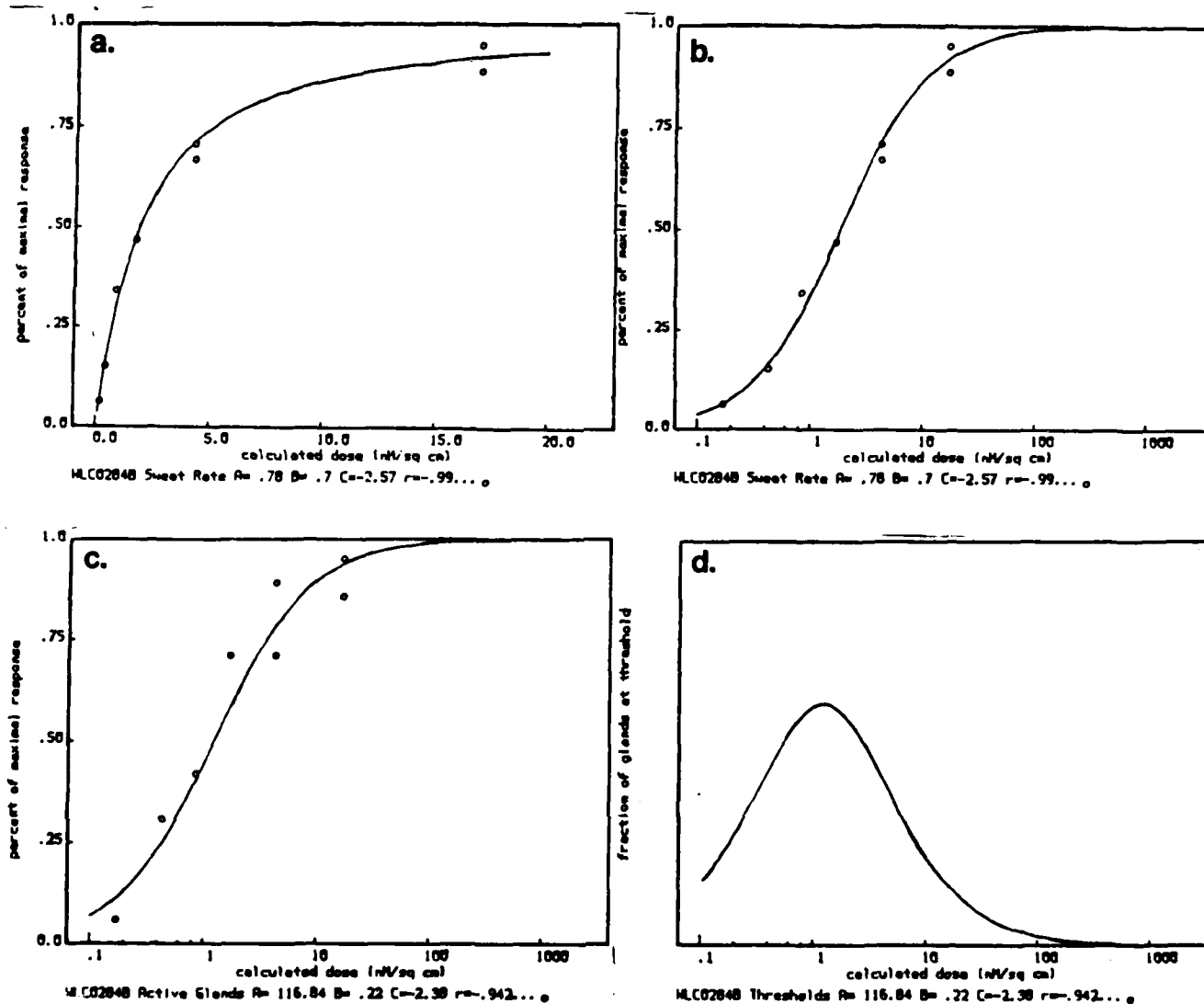


Figure 6. Peak heights and active gland densities from Figure 5 expressed as percent maximal response and replotted as a function of dose. Curves fit by a least-squares hyperbola. a. Peak sweat rate vs. arithmetic dose of mecholyl. b. Peak sweat rate vs. log dose. c. Active gland density (cumulative percent above threshold) vs. log dose. d. Slope of c. showing distribution of thresholds about dose. Mean threshold dose = ED₅₀.

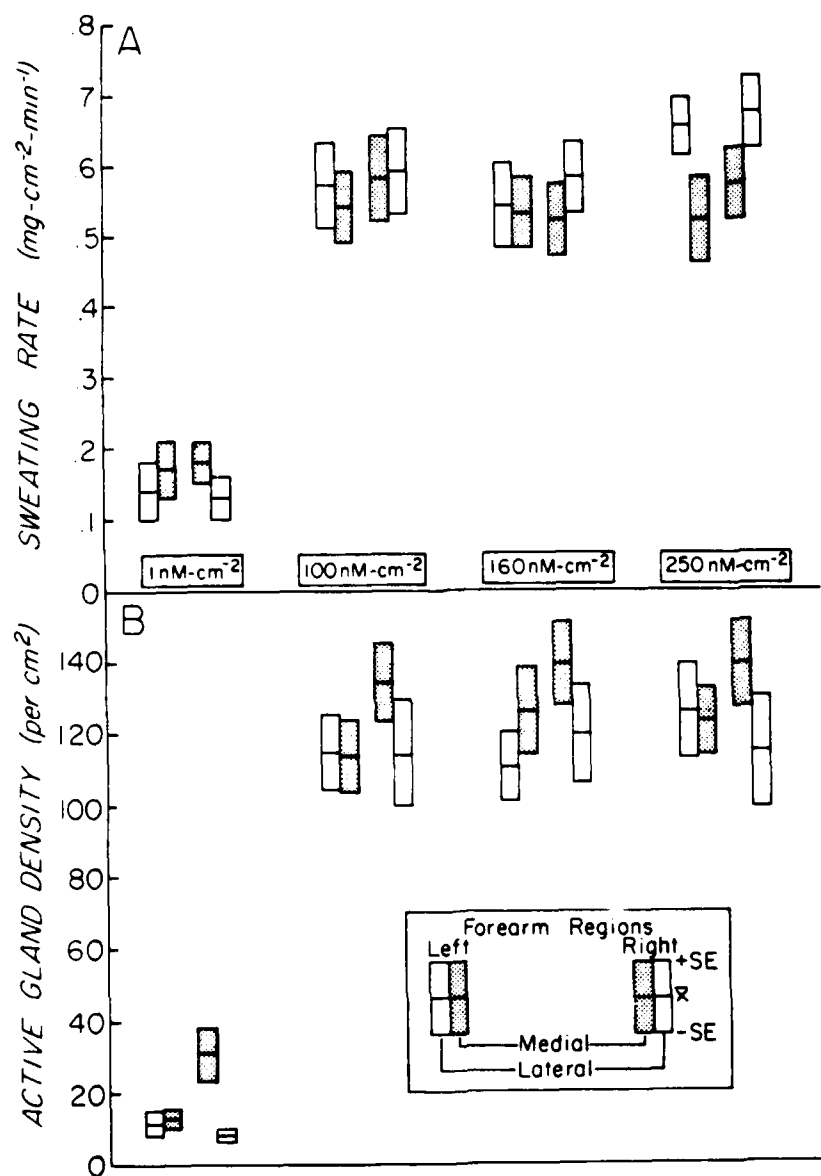


Figure 7. Regional variations in peak sweat rate and active gland density among 7 male subjects. Regions are listed as a, b, c, and d, in order of testing. Active gland density was slightly and consistently higher on region d (inner aspect of right forearm).

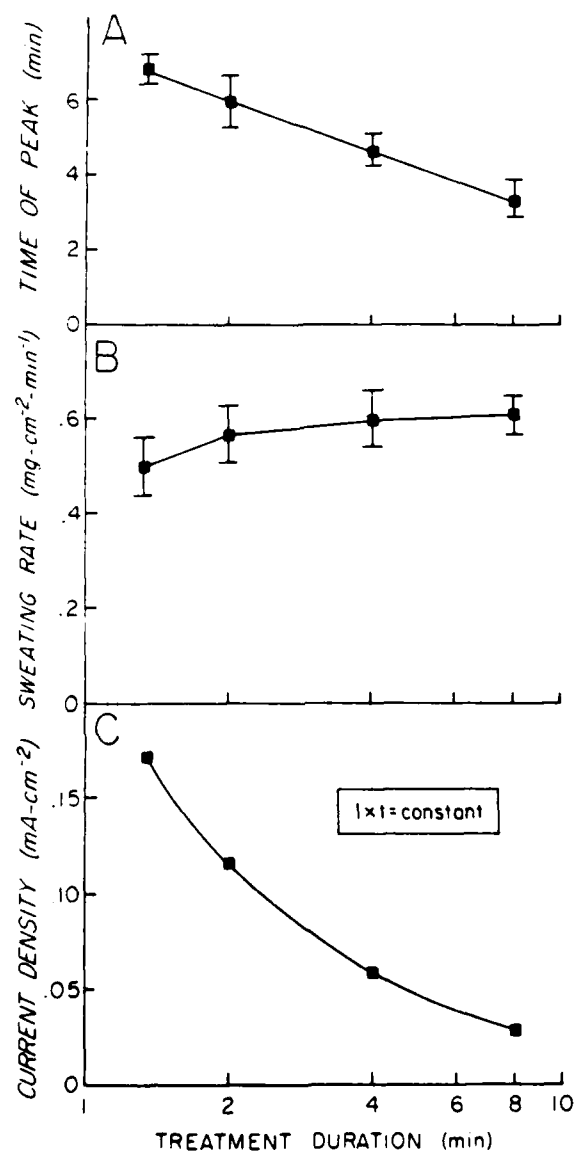


Figure 8. Effect of current and duration on delivered dose. A single midrange dilution dose of mecholyl ($16 \text{ nMol}\cdot\text{cm}^{-2}$) delivered by the 4 different current-duration combinations, each calculated to give a total charge transport of $13.9 \text{ mC}\cdot\text{cm}^{-2}$. Height of peak response was slightly, but not significantly, lower using the highest current-shortest duration combination. Peak sweat rate time was inversely related to the log of iontophoresis duration.

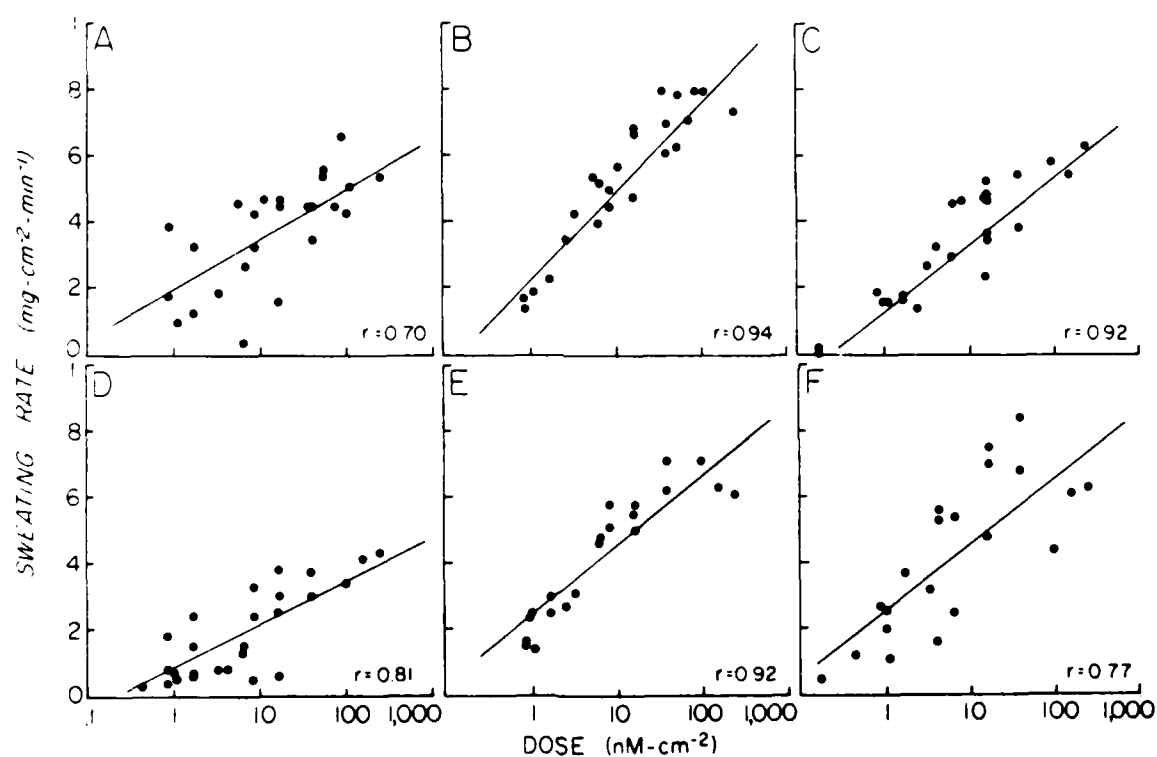


Figure 9. Repeated assays on 6 male subjects over a 3-4 month period using several combinations of current density and duration. Subjects showing most consistent responses within an assay also showed most consistent responses between assays.

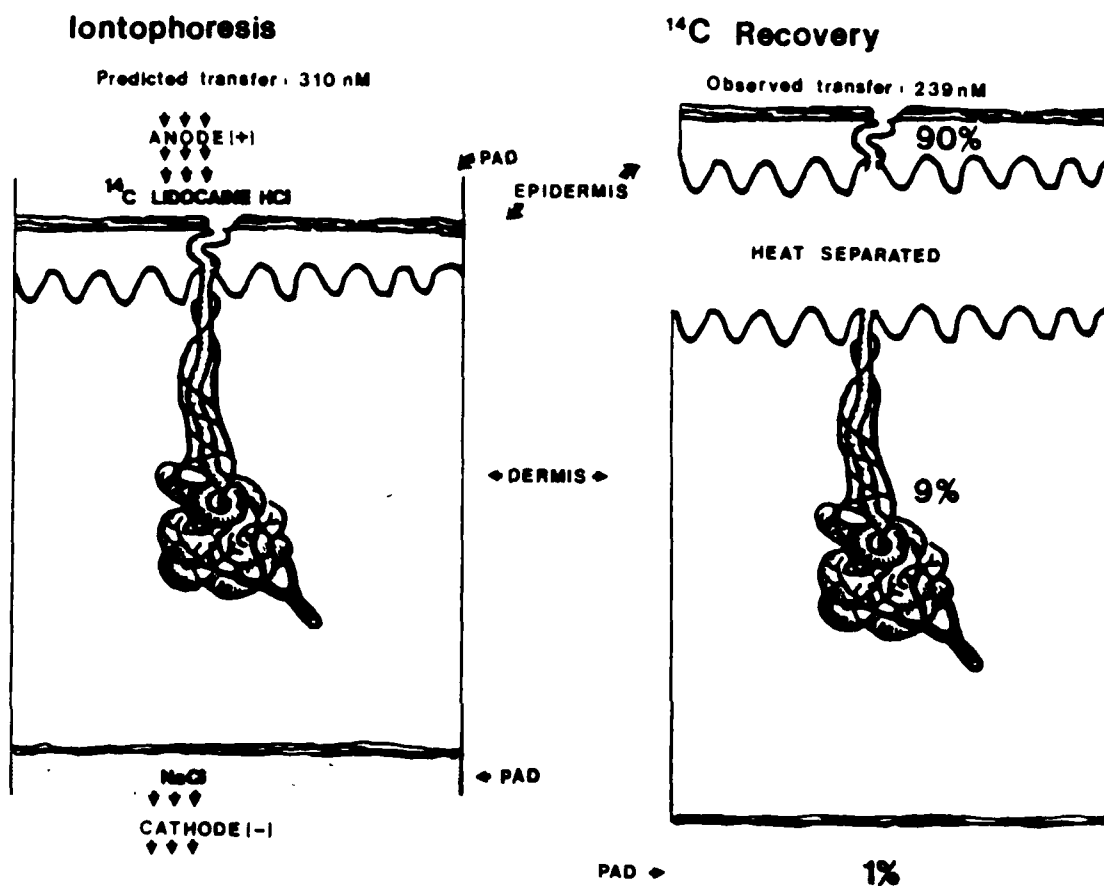


Figure 10. Results of a pilot experiment on the iontophoretic transfer of ^{14}C -labeled lidocaine-HCl through 5 samples of fresh autopsy skin. The molecular weight of lidocaine is similar to that of meholyl, and both should have roughly the same electrophoretic mobility. Most of the label remained in the epidermis; less than 10% of the labeled cations actually reached the dermis.

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) Chubley et al. [1] proposed that measurement of forearm sweat responses to graded intra-dermal injections of acetylcholine can be used as a sensitive index of anticholinergic drug potency. Independently, we developed a human sweat gland assay, differing from theirs in several important respects: It stimulates larger, well-defined areas; it uses non-invasive iontophoresis [2] instead of intradermal injection for administration of mecholyl; it is comprehensive, and measures both active gland density and the time course of sweat production simultaneously on four adjacent test sites [3] instead of only total glands responding; and antagonists can be administered to skin sites by iontophoretic pretreatments and their effects on mecholyl dose-response relationships directly measured. By Faraday's Law, the total dose of ions delivered to skin is proportional to the product of current density and duration of iontophoresis. This defines the highest possible dose, i.e., the condition in which only drug ions are available for transport. In these			
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studies current density and duration were kept constant at 0.132 mA-cm^{-2} and 180 sec, respectively, and the maximum drug transfer was 250 nMol-cm^{-2} . Lesser doses are produced with the same current-time combination by diluting drug solutions with equimolar NaCl solution: The total number of ions transported is always the same but the proportion of drug ions varies. From over 300 individual test replications in more than 35 subjects, we conclude that this assay method can be used safely in normal subjects without peripheral or systemic side effects. Typical mecholyl log dose-response curves are produced for both sweat rate and active gland density over a dose range of 0.4 to 250 nMol-cm^{-2} mecholyl [4].

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